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Activation of Pyruvate Dehydrogenase in Adipose Tissue by Insulin

EVIDENCE FOR AN EFFECT OF INSULIN ON PYRUVATE DEHYDROGENASE PHOSPHATE PHOSPHATASE

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1. The mechanism by which insulin activates pyruvate dehydrogenase in rat epididymal adipose tissue was further investigated. 2. When crude extracts, prepared from tissue segments previously exposed to insulin (2m-i.u./ml) for 2min, were supplemented with Mg^{2+}, Ca^{2+}, glucose and hexokinase and incubated at 30°C, they displayed an enhanced rate of increase in pyruvate dehydrogenase activity compared with control extracts. 3. When similar extracts were instead supplemented with fluoride, ADP, creatine phosphate and creatine kinase, the rate of decrease in pyruvate dehydrogenase activity observed during incubation at 30°C was unaffected by insulin treatment. 4. It is suggested that insulin increases the fraction of pyruvate dehydrogenase present in the tissue in the active dephospho form by increasing the activity of pyruvate dehydrogenase phosphate phosphatase.

Since the discovery by Reed and co-workers (Linn *et al.*, 1969*a*,*b*) that pyruvate dehydrogenase in several mammalian tissues exists both in an inactive phosphorylated form (pyruvate dehydrogenase phosphate) and an active unphosphorylated form (pyruvate dehydrogenase), a great deal of work has been done on factors that might control the degree of phosphorylation and thus the activity of this critical enzyme. In rat adipose tissue, pyruvate dehydrogenase activity has been shown to be subject to hormonal regulation (Jungas, 1970; Denton *et al.*, 1971; Weiss *et al.*, 1971). When insulin or prostaglandin E_1 is added to intact adipose-tissue cells, there is an increase in the assayable pyruvate dehydrogenase activity in tissue extracts (Taylor *et al.*, 1973). The activation of the enzyme results from an increase in the fraction of the enzyme present in the active unphosphorylated form (Coore *et al.*, 1971; Weiss *et al.*, 1971; Taylor & Jungas, 1974). This effect of insulin can be reversed by adrenaline or ACTH‡ (Coore *et al.*, 1971; Jungas, 1971). These hormonal influences are of special significance in the regulation of lipogenesis in rat adipose tissue, since the activity of pyruvate dehydrogenase appears to be one factor limiting the capacity of this pathway (Taylor & Jungas, 1974).

Pyruvate dehydrogenase is unique among enzymes subject to covalent regulation via a phosphorylation–dephosphorylation mechanism in that it is confined to the mitochondrial compartment of the cell. Further, neither the pyruvate dehydrogenase kinase or the pyruvate dehydrogenase phosphate phosphatase appear to be directly affected by cyclic AMP or cyclic GMP (Jungas, 1971; Hucho *et al.*, 1972; Siess & Wieland, 1972; Randle & Denton, 1973). As a first step in exploring the mechanisms by which insulin might influence the degree of phosphorylation of this mitochondrial protein, we have sought to determine whether it is the pyruvate dehydrogenase kinase, the pyruvate dehydrogenase phosphate phosphatase, or both whose activity is modulated by the hormone. We present here data suggesting that the major effect of insulin is to increase the activity of pyruvate dehydrogenase phosphate phosphatase.

Experimental

Methods

Tissue handling. Care of rats and dissection of epididymal adipose-tissue segments was as described previously (Taylor *et al.*, 1973). Segments obtained from two to four rats were pooled for use in each experiment. When the action of insulin was to be studied two pools of segments were formed each containing approx. 1g of tissue. The pools were separately incubated at 37°C in 10ml of Krebs–Henseleit (1932) bicarbonate medium containing one-half the recommended calcium and 2mg of fructose per ml. The gas phase was CO_2+air (5:95). After 30min the segments were transferred to 9ml of fresh medium of the same composition, which had been pre-warmed and pre-gassed. After a second

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‡ Abbreviations: ACTH, adrenocorticotrophin; EGTA, ethanedioxybis(ethylamine)tetra-acetate.

incubation period of 10min there was added 1ml of the same medium with or without insulin to give a final concentration of 1–4m-i.u./ml. From 1 to 4min later the segments were homogenized for assay of pyruvate dehydrogenase. In other experiments insulin or insulin plus adrenaline were present throughout the entire second incubation period.

Assay of pyruvate dehydrogenase phosphate phosphatase. A pool of adipose-tissue segments (1–1.6g) was homogenized by hand in 4vol. of ice-cold medium [10mM-potassium phosphate–1mM-EGTA–1mM-dithiothreitol–0.05% Triton X-100 (pH7.4)] in an all-glass Ten Broeck apparatus. The homogenate was centrifuged at 700*g* for 2min at 2°C and left on ice for a few minutes to permit the fatty layer to solidify. The aqueous infranatant was then recovered and divided into three portions. One portion was used to obtain an initial or zero-time value and was simply kept on ice until being assayed for pyruvate kinase. A second portion was supplemented with 1.25mM-$CaCl_2$ and 5mM-$MgCl_2$, incubated at 30°C for 35–45min, and assayed to reveal the maximum or fully activated pyruvate dehydrogenase content. To the third portion was added 1.25mM-$CaCl_2$, 1.25mM-$MgCl_2$, 10mM-D-glucose and 1unit (μmol/min) of hexokinase/ml. This portion was also incubated at 30°C and samples were withdrawn at suitable intervals and assayed for pyruvate dehydrogenase activity. The increase with time of dehydrogenase activity was taken as a reflexion of pyruvate dehydrogenase phosphate phosphatase activity. Pyruvate dehydrogenase activity was assayed by measuring the evolution of $^{14}CO_2$ from [1-^{14}C]-pyruvate during a 2min period at 37°C as described previously (Taylor *et al.*, 1973). One unit of pyruvate dehydrogenase activity corresponds to 1μmol of CO_2/min at 37°C.

Assay of pyruvate dehydrogenase kinase. Pooled tissue segments were homogenized and centrifuged as described above in a medium containing 10mM-potassium phosphate, 1mM-EGTA, 1mM-dithiothreitol, 1% bovine serum albumin and 1.5mM-$MgCl_2$. A portion of the infranatant was assayed for pyruvate dehydrogenase activity without further treatment. Another portion was supplemented with 8mM-NaF, 8μM-ADP, 4mM-creatine phosphate and 0.24unit (μmol/min) of creatine kinase/ml. It was then incubated at 25°C and samples were withdrawn at suitable intervals for pyruvate dehydrogenase assay. The decline with time of pyruvate dehydrogenase activity was taken as an indication of pyruvate dehydrogenase kinase activity.

Materials

Yeast hexokinase, rabbit muscle creatine kinase and creatine phosphate were obtained from Boehringer (Mannheim) Corp., New York, N.Y., U.S.A. Other suppliers were as described previously (Taylor *et al.*, 1973).

Results

Pyruvate dehydrogenase phosphate phosphatase

When crude extracts of adipose tissue containing EGTA are incubated at 30°C there is little change in their pyruvate dehydrogenase activity. If glucose and hexokinase are added to the extract to deplete it of ATP thereby depriving pyruvate dehydrogenase kinase of an essential substrate, and if Mg^{2+} and Ca^{2+} are added to satisfy the ionic requirements of pyruvate dehydrogenase phosphate phosphatase, there is an immediate rise in pyruvate dehydrogenase activity as shown in Fig. 1. We assume that this rise in activity is the result of enzymic dephosphorylation of pyruvate dehydrogenase phosphate, a process which has been carefully studied in Reed's laboratory (Linn *et al.*, 1972; Hucho *et al.*, 1972).

In support of this assumption we show in Fig. 2 that the rise in dehydrogenase activity under these conditions is sensitive to NaF, a recognized inhibitor of pyruvate dehydrogenase phosphate phosphatase

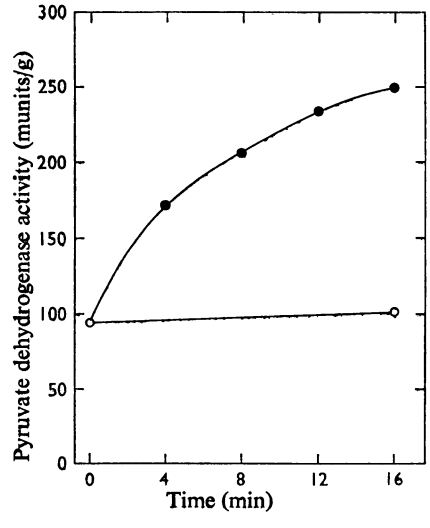

Fig. 1. *Change with time of pyruvate dehydrogenase activity of adipose-tissue extracts*

Adipose-tissue segments were homogenized and assayed for pyruvate dehydrogenase as described under 'Methods' except that maximal dehydrogenase activity was not determined. The time for which the extract was incubated at 30°C before assay is shown on the abscissa. ○, Unsupplemented extract; ●, extract supplemented with Mg^{2+}, Ca^{2+}, glucose and hexokinase as described under 'Methods'. Data points represent averages of triplicate determinations from a single representative experiment.

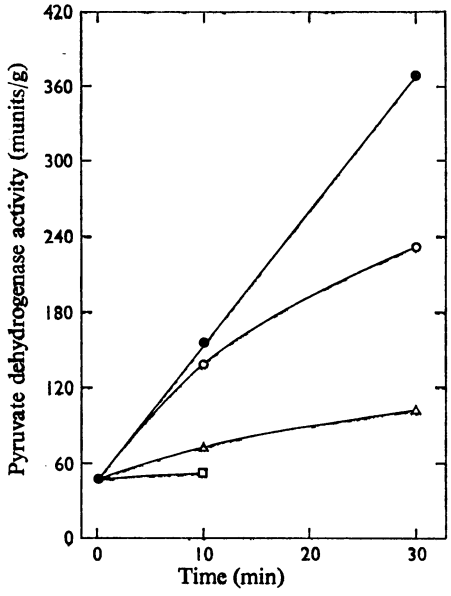

Fig. 2. *Inhibition of pyruvate dehydrogenase activation by fluoride*

An extract was prepared and supplemented with Mg^{2+}, Ca^{2+}, glucose and hexokinase as described under 'Methods'. After the further addition of (●) water, (○) 1mM-NaF, (△) 5mM-NaF, or (□) 10mM-NaF and incubation at 30°C for the times indicated on the abscissa, portions were removed for determination of pyruvate dehydrogenase activity.

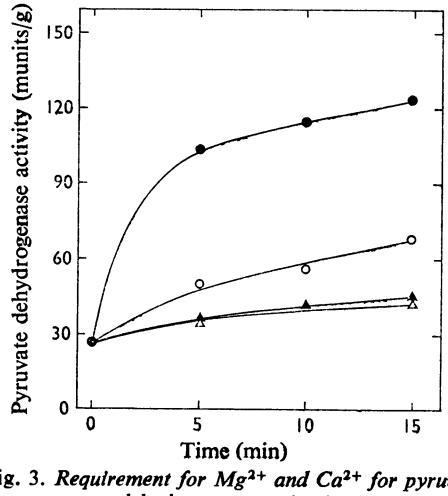

Fig. 3. *Requirement for Mg^{2+} and Ca^{2+} for pyruvate dehydrogenase activation*

An extract was prepared as described under 'Methods' and supplemented with (●) Mg^{2+}, Ca^{2+}, glucose and hexokinase, (○) Mg^{2+}, glucose and hexokinase, (▲) Ca^{2+}, glucose and hexokinase, (△) glucose and hexokinase. After incubation at 30°C as shown on the abscissa portions were removed for assay of pyruvate dehydrogenase activity.

(Hucho *et al.*, 1972). Moreover, both Mg^{2+} and Ca^{2+} are required for activation of the dehydrogenase (Fig. 3) as expected from the known properties of pyruvate dehydrogenase phosphate phosphatase. We have therefore used the rate of increase of dehydrogenase activity under these conditions as a measure of the pyruvate dehydrogenase phosphate phosphatase in the extracts.

Effect of insulin on pyruvate dehydrogenase phosphate phosphatase

The enzyme assay described above was used to determine whether exposure of tissue segments to insulin would alter phosphatase activity. In the experiment summarized in Fig. 4, one of two paired pools of tissue segments was exposed to 1m-i.u. of insulin/ml for 2min, a period too brief to allow a significant rise in pyruvate dehydrogenase activity to occur. The tissues were homogenized and the pyruvate dehydrogenase activity of each extract was monitored during incubation as shown in the Figure. The dehydrogenase activity of the extract prepared from the insulin-treated tissue rose about 65% more rapidly than in the control. Note that the initial dehydrogenase activity of the extracts was about the same, that is, there was no difference in the concentration of the substrate of the

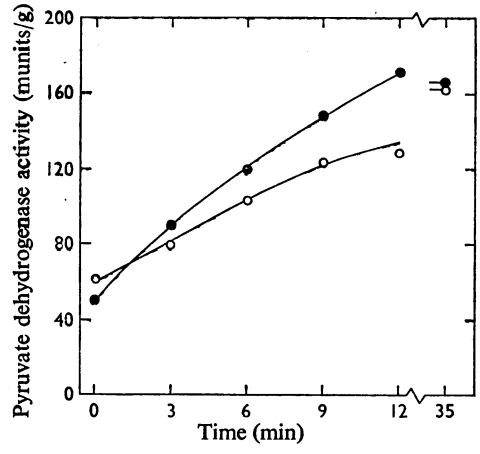

Fig. 4. *Effect of insulin on activation of pyruvate dehydrogenase*

Pools of adipose-tissue segments were incubated as described under 'Methods'. Insulin (1m-i.u./ml) was added to one pool of segments during the final 2min of incubation. Extracts were then prepared and assayed for pyruvate dehydrogenase activity as described in the text. Data points represent averages of duplicate determinations from a single representative experiment. ○, Control tissue; ●, insulin-treated tissue.

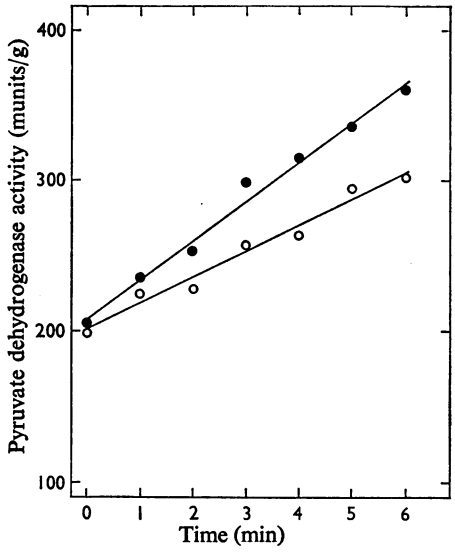

Fig. 5. *Effect of insulin at early time-periods*

Data from two experiments have been combined as the results were similar in each. Pools of tissue segments were incubated as described under 'Methods'. Insulin was then added (2m-i.u./ml for 2min in one case, 4m-i.u./ml for 3min in the other), and the tissue was homogenized and assayed for pyruvate dehydrogenase activity. The maximal pyruvate dehydrogenase activity observed after 45min was 560 in the control and 570 for insulin-treated tissue. The lines have been drawn by the method of least squares. ○, Control tissue; ●, insulin-treated tissue.

phosphatase in the two extracts. A portion of each extract was incubated for 35min before assay so as to achieve maximal activation of the dehydrogenase. At this time also there was no difference in dehydrogenase activity between the control and insulin-treated extracts. It thus appears that the brief exposure to insulin caused a rise in the pyruvate dehydrogenase phosphate phosphatase activity but no change in the total pyruvate dehydrogenase content of the extracts.

If this interpretation is correct the most favourable time to observe the insulin effect should be during the first moments of incubation of the extract, before the pyruvate dehydrogenase phosphate phosphatase has substantially depleted its substrate. Data from two experiments with rapid sampling are shown in Fig. 5. The magnitude of the insulin effect appears similar to that seen previously.

A statistical evaluation of this effect of insulin is given in Table 1. On the average the insulin-treated extracts showed 65% greater phosphatase activity during the initial 5min period of incubation. During this interval the extracts of control tissues attained 27±4% of their maximal pyruvate dehydrogenase activity, and extracts of insulin-treated tissues reached 43±4%.

Effect of adrenaline on pyruvate dehydrogenase phosphate phosphatase

In experiments similar to those just described we could observe no effect of adrenaline at concen-

Table 1. *Effect of insulin in the activation of pyruvate dehydrogenase*

Pyruvate dehydrogenase activity was measured in fresh extracts ('zero time'), after 5min of incubation at 30°C in the presence of Mg^{2+}, Ca^{2+}, glucose and hexokinase, and after maximum activation achieved as described under 'Methods'. Statistical comparisons used Student's two-tailed *t* test for paired values. Data are averages±S.E.M. for six experiments. N.S., not significant.

	Pyruvate dehydrogenase activity (munits/g)				
Additions	Zero time	5min	5min increment	Maximal	Percentage of maximal activation achieved in 5 min
Control	101±34	156±64	55±11	317±80	27±4
Insulin	97±45	188±51	91±16	324±84	43±4
P	N.S.	0.015	0.003	N.S.	0.001

Table 2. *Inhibition by adrenaline of the action of insulin*

Pyruvate dehydrogenase activity was measured in fresh extracts ('zero time'), after either 6 or 10min of incubation at 30°C in the presence of Mg^{2+}, Ca^{2+}, glucose and hexokinase, and after maximum activation achieved as described under 'Methods'. Statistical comparisons used Student's two-tailed *t* test for paired values. Data are averages±S.E.M. for five experiments. N.S., not significant.

	Pyruvate dehydrogenase activity (munits/g)				Percentage of maximal activation achieved in	
Additions	Zero time	6min	10min	Maximal	6min	10min
Insulin (2m-i.u./ml)	83±10	105±9	120±10	129±13	49±6	72±11
Insulin plus adrenaline (8 μM)	79±9	92±11	108±12	132±15	25±3	43±9
P	N.S.	0.03	0.05	N.S.	0.01	0.03

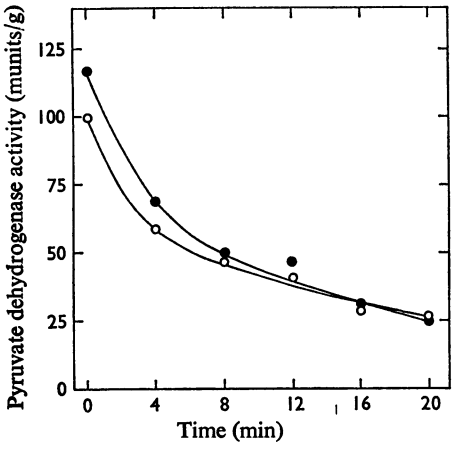

Fig. 6. *Effect of insulin on inactivation of pyruvate dehydrogenase*

Pools of tissue segments were incubated as described under 'Methods'. Insulin (2m-i.u./ml) was added to one pool of segments during the final 1.5min of incubation. Extracts were prepared and assayed for pyruvate dehydrogenase activity as described in the text. Data points are averages of duplicate determinations from a single representative experiment. ○, Control tissue; ●, insulin-treated tissue.

trations up to 10μM on pyruvate dehydrogenase phosphate phosphatase activity. However, it was possible to block the increase in phosphatase activity caused by insulin by the simultaneous addition of adrenaline. As detailed in Table 2, extracts of tissue exposed to both insulin and adrenaline for 10min had lower phosphatase activity than extracts of paired segments exposed only to insulin. The percentage of maximum activation achieved in 6min was nearly halved by the presence of adrenaline and now closely resembled that seen in extracts of control tissues.

Pyruvate dehydrogenase kinase

If crude extracts of adipose tissue are supplemented with NaF to block pyruvate dehydrogenase phosphate phosphatase, and provided with an ATP-generating system, incubation at 30°C leads to an abrupt fall in pyruvate dehydrogenase activity. From these requirements, we assumed that the declining dehydrogenase activity is the result of an enzymic phosphorylation of pyruvate dehydrogenase, as studied in detail in Reed's laboratory (Linn *et al.*, 1972; Hucho *et al.*, 1972). Using this system as an assay for pyruvate dehydrogenase kinase in tissue extracts we have been unable to demonstrate any effect of insulin or adrenaline treatment of tissue segments. An example of this type of experiment is shown in Fig. 6.

We have also studied pyruvate dehydrogenase kinase activity in extracts first incubated with an excess of Mg^{2+} and Ca^{2+} so as to generate maximal pyruvate dehydrogenase activity. Subsequent addition of fluoride and an ATP-generating system causes an abrupt fall in dehydrogenase activity, but hormonal treatment of the tissue is again without effect on the apparent kinase activity of the extracts.

Discussion

Mechanisms for changing enzyme activity *in vivo* may be divided into two broad classes termed 'rapidly' and 'slowly' reversible. Only changes of the slowly reversible type are sufficiently long-lived to be observed after the tissue contents have been diluted by homogenization. The data presented here indicate that pyruvate dehydrogenase phosphate phosphatase is activated by insulin in a slowly reversible manner. Although it is tempting to identify such slowly reversible changes with covalent modifications, other mechanisms must also be considered. For example, a ligand which is very tightly bound to the enzyme might dissociate from the enzyme so slowly as to allow its effect to be observed for extended periods after dilution. Alternatively, insulin could cause the accumulation of a substance with the property of catalysing conformational transitions of the enzyme. Dilution would freeze the enzyme in one conformation by removing the catalyst. This type of regulation in which small molecules frequently serve as catalysts has been termed 'pre-conditioning' (Alpers *et al.*, 1971; Paulus & Alpers, 1971).

At present there are three factors known to affect pyruvate dehydrogenase phosphate phosphatase activity: Mg^{2+} (Linn *et al.*, 1969*a*); Ca^{2+} (Denton *et al.*, 1972; Hucho *et al.*, 1972: Siess & Wieland, 1972); and free citrate (Taylor & Halperin, 1973). Whether any of these factors is involved in the slowly reversible activation of pyruvate dehydrogenase phosphate phosphatase by insulin is not known. The activation is not readily explained by tight binding of calcium. In the present experiments, 1mM-EGTA was added to the homogenizing fluid for the purpose of chelating tissue calcium. Moreover, an excess of calcium was added during the phosphatase assay and yet elevated activity was observed. In other experiments not shown here, it was found that the addition of 1mM-EGTA arrested pyruvate dehydrogenase phosphate phosphatase activity very rapidly in adipose-tissue extracts. Thus EGTA does successfully compete with the enzymes for calcium. We do not yet know how rapidly citrate dissociates from pyruvate dehydrogenase phosphate phosphatase.

The fact that the activation of the phosphatase by insulin could be blocked by adrenaline suggests that cyclic AMP may be at least indirectly involved in this

regulatory system. Since it is well known that lipolysis and hence cellular concentrations of free fatty acids are affected by both adrenaline and insulin in this tissue, possible effects of fatty acids and their CoA thioesters on pyruvate dehydrogenase phosphate phosphatase deserve careful study.

An activation of pyruvate dehydrogenase phosphate phosphatase by insulin has been reported previously by Sica & Cuatrecasas (1973). These workers used exogenous pyruvate dehydrogenase phosphate as substrate. The results of Sica & Cuatrecasas (1973) therefore indicate that the effect of insulin is not merely to change pyruvate dehydrogenase phosphate so as to make it a better substrate for the phosphatase. Rather, there must be an effect of insulin on pyruvate dehydrogenase phosphate phosphatase itself.

No slowly reversible effect of insulin on pyruvate dehydrogenase kinase could be detected in the present studies. Thus we tentatively rule out any covalent modification of this enzyme. Regulation of pyruvate dehydrogenase kinase undoubtedly does play an important role in the physiological regulation of pyruvate dehydrogenase and the intramitochondrial ATP/ADP ratio is probably a major factor determining the activity of this enzyme (Taylor *et al.*, 1975). In many tissues the major role of pyruvate dehydrogenase appears to be in the generation of ATP by providing acetyl-CoA for oxidation via the citric acid cycle. Thus regulation of pyruvate dehydrogenase activity by the mitochondrial ATP/ADP ratio seems most appropriate. However, in adipose tissue, a large fraction of the acetyl-CoA generated by pyruvate dehydrogenase is converted into fatty acids. Moreover, lipogenesis occurs at high rates at times when the cellular concentrations of ATP are high, for example in the presence of excess of glucose and insulin (Denton *et al.*, 1966; Flatt, 1970). If the mitochondrial ATP/ADP ratio is also high under these conditions, adipose tissue would require a mechanism for overcoming the feedback regulatory system based on this ratio. Such a mechanism appears to be provided by insulin acting on pyruvate dehydrogenase phosphate phosphatase. The activation of this enzyme would allow pyruvate dehydrogenase phosphate to be dephosphorylated despite an unfavourable ATP/ADP ratio, and thus permit acetyl-CoA to be fed rapidly into the lipogenic pathway. It would therefore not be surprising if the effects of insulin on glucose transport and on pyruvate dehydrogenase phosphate phosphatase were closely related mechanistically.

A similar situation exists in liver where lipogenesis is also an important process. However, to date, all our attempts to demonstrate an activation of liver pyruvate dehydrogenase by insulin have been without success. Glucose utilization in liver is not nearly so dependent on insulin as in adipose tissue (Cahill *et al.*, 1958; Williams *et al.*, 1968), and apparently the increased flux through pyruvate dehydrogenase needed for lipogenesis is accomplished simply via elevations in liver pyruvate concentrations (Patzelt *et al.*, 1973).

Severson *et al.* (1974) were unable to observe any action of insulin on pyruvate dehydrogenase phosphate phosphatase activity and their results thus differ from the present finding and the earlier report of Sica & Cuatrecasas (1973). We do not understand the reasons for these discrepancies. In experiments similar to those summarized in Table 1, Severson *et al.* (1974) reported a pyruvate dehydrogenase phosphate phosphatase activity of 26–28 (as the increase in endogenous pyruvate dehydrogenase activity, munits/g during 5min) with or without prior exposure to insulin (5m-i.u./ml for 5min). Our values are twice as high in control tissue and more than three times higher with insulin-treated tissue. Among other minor differences in procedure it should be noted that Severson *et al.* (1974) froze the tissue during their extraction procedure, used a tenfold greater concentration of potassium phosphate in their extraction medium, and used a motor-driven tissue grinder. In our experience the composition of the medium used for tissue homogenization may be varied considerably without altering the observed insulin effect. For example, we have substituted 10mM-sucrose for the 10mM-potassium phosphate, replaced the 0.05% Triton X-100 with 1% bovine serum albumin and replaced EGTA with EDTA without significantly modifying the insulin effect. In three experiments no magnesium or calcium was added during the phosphatase assay. This greatly diminished the rise in pyruvate dehydrogenase activity during incubation of the extracts, but the insulin effect was still observable. The control extracts now attained only $3.3\pm1.7\%$ of their maximal activation during the initial 5min of incubation whereas the extracts of insulin-treated tissue reached $6.7\pm1.2\%$. In a series of 12 experiments the length of exposure to insulin was varied between 1 and 5min and in each instance an effect of insulin was noted. We are thus unable to account for the negative findings of Severson *et al.* (1974).

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